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Biocontrol of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in Meat by Using Phages Immobilized on Modified Cellulose Membranes▽

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The ability of phages to specifically interact with and lyse their host bacteria makes them ideal antibacterial agents. The range of applications of bacteriophage can be extended by their immobilization on inert surfaces. A novel method for the oriented immobilization of bacteriophage has been developed. The method was based on charge differences between the bacteriophage head, which exhibits an overall net negative charge, and the tail fibers, which possess an overall net positive charge. Hence, the head would be more likely to attach to positively charged surfaces, leaving the tails free to capture and lyse bacteria. Cellulose membranes modified so that they had a positive surface charge were used as the support for phage immobilization. It was established that the number of infective phages immobilized on the positively charged cellulose membranes was significantly higher than that on unmodified membranes. Cocktails of phages active against *Listeria* or *Escherichia coli* immobilized on these membranes were shown to effectively control the growth of *L. monocytogenes* and *E. coli* O157:H7 in ready-to-eat and raw meat, respectively, under different storage temperatures and packaging conditions. The phage storage stability was investigated to further extend their industrial applications. It was shown that lyophilization can be used as a phage-drying method to maintain their infectivity on the newly developed bioactive materials. In conclusion, utilizing the charge difference between phage heads and tails provided a simple technique for oriented immobilization applicable to a wide range of phages and allowed the retention of infectivity.

Recently, phages have received regulatory approval for use as a safe food additive in certain food products to enhance food safety (8). Given that phage can be efficacious for the biocontrol of food-borne pathogens, application strategies should be optimized to be the most convenient, most economical, and least invasive to the process itself (12). Interventions using phages can be performed at different or even multiple points in the food-processing facility or throughout the food chain to enhance the control process and to reduce the potential for the development of phage resistance (9). It has been suggested that phages can be added by dipping or spraying or as a liquid to a large volume of food. These methods may not be ideal, as they could be wasteful and lead to the potential inactivation of the phage particles as a consequence of the inclusion of other materials within the wash fluid, such as residues of sanitizers that are used to clean surfaces of processing areas. Moreover, if the phage-containing fluids themselves contain nutrients that support bacterial growth, then the potential for the bacterial evolution of phage resistance exists. Thus, when phages are added directly to a batch of food, two major problems may be encountered: the dilution of phages and the evolution of bacterial resistance. The addition of large numbers and volumes of phages using phage cocktails and the regular disinfection of the equipment using effective protocols might help to overcome these problems (12). These problems also may be overcome by using immobilized phage. This will ensure that phages are applied and retained near the surface that is being treated, thereby avoiding excessive phage waste.

Immobilized biologically active materials are of great importance to industry and research. The selection of the immobilization method and support depends on the nature of the bioactive material and on the application itself (16, 18, 34). The potential use of a phage-based biosorbent to detect, concentrate, and identify target bacteria has been reported in several studies (10, 42). In one approach, physical adsorption has been used recently to immobilize filamentous and *Podoviridae* phages on gold surfaces of surface plasmon resonance (SPR) sensors and glass substrates to be used as a recognition element for many targeted pathogens (2, 13, 26). In an earlier study, *Salmonella* cells were captured from food matrices using *Salmonella*-specific phage passively immobilized on polystyrene, but this resulted in a low capture efficiency (3).

Chemical biotinylation of the phage head has been used to immobilize phages on streptavidin-coated magnetic beads, but again this system only captured low numbers of *Salmonella enteritidis* cells in foods (37). The orientation and/or the inactivation of the phage may have played a role in the low capture efficiency. The site-specific immobilization of phages was suggested as a way to orientate the immobilized phages in such a way as to have tail fibers free and, thereby, increase the efficiency of the capture of the target bacteria. The genetic modification of wild-type T4 phage through a phage display technique has been used to give well-oriented T4 phage particles when immobilized on cellulose

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TABLE 1. Phages and bacterial strains used for phage propagation, immobilization, and biocontrol experiments

Phage	Bacterial host	CRIFS[a] culture collection no.	Strain designation
SenS-AG11	*Salmonella* Enteritidis	C417	En-2588
LinM-AG8	*Listeria innocua*	C505	
LmoM-AG13	*Listeria monocytogenes*	LJH391	4b
LmoM-AG20	*Listeria monocytogenes*	C519	1/2b
SboM-AG3	*Shigella boydii*	C 865-2	
EcoM-AG2	*E. coli* O126:H8	C761	O126:H8, EC 910061
EcoM-AG3	*E. coli* O126:H8	C761	O126:H8, EC 910061
EcoM-AG10	*E. coli* O157:H7	C899	O157:H7, ATCC 43888

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membranes or streptavidin-coated magnetic beads, which resulted in a high capture efficiency for *Escherichia coli* cells (38). However, this protocol was initially laborious and costly even with T4 phage, which is one of the better-characterized phages with a fully sequenced and annotated genome. In addition, these modified phages exhibited a lower burst size and less infectivity than wild-type T4 (24, 38). Therefore, alternative simple protocols are needed to immobilize phages in the right orientation that can be applied to newly isolated phages with known structures but that have not been fully sequenced.

One approach to immobilization may be to use electrostatic interaction between the phage and the support matrix. Interestingly, it was reported that the net charge on most viruses is negative, and the whole T4 phage (capsid, tail, and fibers) has an isoelectric point close to 4 (1). Furthermore, the same study suggested that capsids acquire a negative overall charge above pH 4. In an earlier study, the T7 phage head was suggested to be responsible for the overall negative charge of the phage, and the tail fibers could be positively charged (35).

Cellulose is the most abundant natural polymer on earth and can be considered an attractive matrix for immobilization, mainly because of its combination of excellent physical properties and low price. Moreover, cellulose can be easily manufactured and modified according to the application (22). Regenerated cellulose membranes were chosen as the substrate for phage immobilization, since cellulose materials are cost-effective and environmentally friendly (28). Different bioactive materials, such as nitrifier and denitrifier bacteria (31), *Rhodobacter capsulatus* (33), glucoamylase enzyme (40), bacteriocins (23, 41), and antimycotic agents (6), have been immobilized on cellulose and were applied successfully in several medical, environmental, and food applications.

In a previous study, we postulated that the inherent charge characteristics of phages can be utilized to anchor them in the right orientation to appropriately modified surfaces, which then could serve as anti-bacterial agents (4). In this paper, we report the study of the charge difference between phage heads and tails and the possibility of using charge difference to immobilize two different phage cocktails onto regenerated cellulose film coated with a cationic polymer, polyvinylamine, to control *E. coli* O157:H7 and *Listeria monocytogenes* in raw and ready-to-eat (RTE) meat, respectively.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and cultural conditions. Previously isolated and characterized phages and their susceptible host strains of *E. coli*, *Listeria* spp., *Salmonella enterica* serovar Enteritidis, and *Shigella boydii* (selected from the Canadian Research Institute for Food Safety [CRIFS] Culture Collection at the University of Guelph) were used in this study (Table 1). Wild-type T4 phage (ATCC 11303B4) was included in the dryness experiment, and *E. coli* B (ATCC 11303) was used for its propagation. T4 mutant strains 23amH11 and 11amB255 (10^- and 23^-, respectively), which have an amber mutation in gene 23 and gene 10 to produce only heads or only tails with tail fibers, respectively, and their propagating suppressor host bacterium (*E. coli* CR63) were kindly provided by Fumio Arisaka, Tokyo Institute of Technology, Tokyo, Japan. Ampicillin-resistant/bioluminescent strain *E. coli* O157:H7 (*amp*::*lux*) (C918) from the CRIFS Culture Collection at the University of Guelph was used to test the infectivity of immobilized *E. coli* phages and to artificially inoculate raw meat.

Tryptic soy broth (TSB), tryptic soy agar (TSA), tryptose soft agar (TSB plus 0.4% agarose), and *Listeria* selective agar base (Oxford Formulation) (Difco Laboratories, Detroit, MI) were used in this study to grow the host bacteria, propagate the phages, and count *Listeria monocytogenes* cells after challenge experiments, respectively. The synthetic minimal medium M9A (10% salt mixture [7% Na_2HPO_4, 3% of KH_2PO_4, 0.5% NaCl, 1% NH_4Cl], 0.4% glucose, 0.1% $CaCl_2$ [0.01 M], and 0.1% $MgSO_4$ [0.1 M]) supplemented with a 5% mixture of Casamino Acids (Fisher Scientific, Mississauga, Canada) was used for the production of T4 heads, tails, and tail fiber components from a T4 mutant. Ampicillin (100 μg/ml) was added to TSB and TSA to grow and count ampicillin-resistant *E. coli* O157:H7 (*amp*::*lux*) strain C918.

For the propagation and titer determination of *Listeria* phages, all media were supplemented with filter-sterilized 1.25 mM $CaCl_2$ per liter of the medium after sterilization. The propagation and enumeration of phages were performed using the soft-agar overlay technique as previously described (32). The three phages against *L. monocytogenes* or *E. coli* O157:H7 were mixed together to form the phage cocktails that were used in the immobilized form for the biocontrol experiments. The phage cocktail lysate was stored at 4°C.

Modification of the cellulose membranes. Regenerated cellulose membranes were purchased as dialysis membranes from Spectrum Laboratories, Inc. (Spectra/Por 4 product no. 132709;12-kDa molecular mass cutoff; Spectrum Laboratories, Inc., Rancho Dominguez, CA). The membranes were cut into round pieces with a diameter of 4 cm. The obtained membranes were boiled for 4 to 6 h in distilled water (during which time the water was changed at least three times) to remove any preservative. Afterwards, the cellulose membranes were thoroughly rinsed with water and stored in distilled water at 4°C. Polyvinylamine samples with molecular masses of 1,500 kDa were obtained from BASF Canada, Inc. (Mississauga, Canada). To ensure the complete hydrolysis of the parent poly(N-vinyl formamide), they were treated further under nitrogen purge with 5% NaOH at 70°C for 48 h to remove residual formamide groups. The polymers were dialyzed against water for 10 days and subsequently were freeze dried.

The membranes were modified by adding 0.5% (wt/vol) polyvinylamine polymer dissolved in 0.005 M NaCl at pH 10 for 10 min to produce a positively charged cellulose membrane surface. The polymer-saturated membranes were rinsed with pH 10 salt solution for 5 min to remove unadsorbed polymer.

Immobilization of phages on cellulose membranes. A phage cocktail consisting of the three *E. coli* O157:H7 phages was added to both the positively charged membranes and unmodified membrane. Five milliliters from the phage cocktail at different concentrations (approximately 10^3, 10^5, 10^7, and 10^9 PFU/ml) was added to each membrane and incubated overnight at 4°C with gentle shaking. The produced phage-carrying membranes were labeled M-3, M-5, M-7, and M-9, according to the titer of the phage cocktail initially added to them. The membranes were removed, and the amount of the remaining phages in the supernatant was determined by serial dilution and an overlay technique to determine the

amount of phages deposited on the membranes. The number of the captured phages was calculated by subtraction. The membranes were washed three times in 5 ml phage buffer prior to use.

Investigating the infectivity of the immobilized phages. The infectivity of phages on the membranes was determined by two approaches: an overlay technique and bioluminescence assays. In the overlay technique, the membranes were placed over a top agar layer inoculated with the host bacterium (*E. coli* O157:H7 *amp*::*lux*) and incubated overnight at 25°C before counting the plaques that had developed underneath the membranes. The other approach involved detecting the effect of these membranes on the bioluminescence produced by a luminescent strain of *E. coli* O157:H7 (*amp*::*lux*). Both the phage-carrying and untreated membranes were added to 4 ml of broth medium inoculated with two different concentrations of the indicator bacterium (about 10^3 and 10^5 CFU/ml) in a Corning Costar sterile 6-well flat-bottomed polystyrene microtiter plate (not prepared by the manufacturer for tissue culture work) (Corning Inc., Corning, NY). Each treatment was performed in triplicate along with controls of bacteria, media, and phage buffer only. The plates were incubated at 25°C, and the bioluminescence was measured every hour for 12 h by using a Victor^2 1420 Multilable Counter (Wallac Oy, Turku, Finland).

Investigating the overall charge difference between phage head and tail structures. T4 heads and tails with tail fibers were prepared by using T4 mutants 10^- and 23^-, respectively, and then the Zeta potential was measured to determine the overall charge on each preparation. The T4 mutant strains were first propagated in the suppressor *E. coli* host strain CR63 to obtain the infectious phage using the overlay technique (32). The infectious phages then were used to produce noninfectious capsids and tails with tail fibers in the nonsuppressor host *E. coli* strain, *E. coli* B, by a modification of a previous procedure (1). Briefly, the *E. coli* B cells were grown in 1 liter of M9A broth until an optical density at 600 nm (OD_{600}) of around 0.2 was obtained, and then they were infected with 10^- or 23^- T4 phage mutant at a multiplicity of infection (MOI) of around 1. The cells were superinfected with the same phage mutants after 5 and 10 min. The whole mixture was incubated for 3 h at 37°C with gentle shaking. Cells were spun down and resuspended in 10 ml phage buffer. Chloroform (1 ml), DNase I (20 μg/ml), and RNase I (50 μg/ml) (Invitrogen Canada, Burlington, Canada) then were added to the cell suspension and shaken at 37°C for 1 h. The cell debris was removed after spinning at 5,000 × *g* for 30 min. The produced supernatant was washed three times by centrifugation at 16,000 × *g* for 1 h at 4°C to remove the particles, which then were resuspended in 500 μl Milli-Q sterile water. The overall charges of the heads and tails-with-tail-fibers preparations were determined by using a Zeta potential analyzer (Zetasizer Nano; Malvern Instruments, Worcestershire, United Kingdom). Two separate preparations were made from each mutant, and five Zeta potential measurements were performed for each preparation.

Potential application of the immobilized phage cocktails to control food-borne pathogens on meat surfaces. *Listeria* and *E. coli* phage cocktails were immobilized on cellulose membranes using an initial phage titer of approximately 10^9 PFU/ml for addition to the positively charged membranes. The immobilized phages were applied to ready-to-eat, oven-roasted turkey breast and raw beef inoculated with *L. monocytogenes* (C391) and *E. coli* O157:H7 (*amp*::*lux*) (C918) strains, respectively. The luminescent strain of *E. coli* O157:H7 was used to enable direct plating and reduce problems posed by the contamination of the plates with background microflora. Meat was purchased on the initial day of the experiment from local grocery stores and initially screened for contamination with the targeted bacteria. Twenty-five grams of meat was removed and weighed aseptically, placed in sterile petri dishes, and then preequilibrated to the desired temperature. An overnight culture of each bacterial strain was diluted to approximately 10^3 CFU/ml. A 100-μl aliquot was spotted onto the surface of each piece of meat and allowed to attach for 10 min at room temperature. This was followed by the addition of the matrix containing immobilized phages to cover the contaminated surface of the meat. Phage-free, positively charged membranes and unmodified membranes exposed to phage were used as controls. For *Listeria*-containing samples, meat was incubated at 25, 10, and 4°C for 2, 4, and 15 days, respectively, under aerobic, modified atmosphere packaging (MAP), and vacuum packaging conditions. The modified atmosphere was composed of approximately 1% O_2, 13% CO_2, and 86% N_2, which was generated using an AnaeroGen sachet in a sealed anaerobic jar (Oxoid, Fisher Scientific, Mississauga, Canada). The vacuum packaging was done using a Komet vacuum packaging machine (Komet Maschinenfabrik GmbH, Plochingen, Germany) at a vacuum of 1.0 bar. The meat was packed in sterile polyethylene bags of 65 μm thickness (Seward Laboratory Systems Inc., Bohemia, NY) before being placed in the vacuum machine. *E. coli*-containing meats were incubated only aerobically at the same temperatures and incubation periods.

Counts were performed for both hosts after 6, 12, 24, and 48 h for samples incubated at 25°C; 1, 2, 3, and 4 days for samples incubated at 10°C; and 1, 3, 6, 9, 12, and 15 days for those incubated at 4°C. In all cases, the total viable bacterial counts (CFU/g) were determined immediately after the addition of bacteria and phage. Triplicate samples were analyzed at each sampling time when the membrane was removed and the meat was transferred to a WhirlPak bag (Nasco, Fort Atkinson, WI) containing 225 ml of 0.85% saline solution. The sample was stomached for 1 min at 200 rpm (Seward Blender Stomacher 400 circulator; Seward Laboratory Systems Inc., NY). The liquid portion was transferred to a sterile centrifuge tube and serially diluted in sterile 0.85% saline solution. One hundred microliters was plated in duplicate on Oxford agar plates and TSA-plus-ampicillin plates for *L. monocytogenes* and *E. coli* O157:H7 counts, respectively, and incubated at 37°C for 48 h in the case of *L. monocytogenes* until typical *Listeria* colonies could be enumerated and for 24 h for the *E. coli* O157:H7 strain. The bioluminescent colonies of the *E. coli* O157:H7 strain were counted using a NightOwl II LB 983 Molecular Imager (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany).

In another approach to check the efficiency of the immobilized *E. coli* phages to control the growth of *E. coli* O157:H7, the immobilized phages were added to the artificially contaminated raw meat and incubated at 4 or 10°C for 1 week and then at 30°C for 16 h. Afterwards, the treated samples along with the controls covered with phage-free, positively charged membranes were examined using the NightOwl to detect the bioluminescence emitted from their surfaces.

Effect of dryness on stability of phages. The stability of phages after drying was investigated using five different phages: four *Myoviridae* phages (wild-type T4, EcoM-AG2, SboM-AG3, and LinM-AG8) and one *Siphoviridae* phage (SenS-AG11). Two hundred microliters from each phage lysate of known titer was left to be air dried for 3 and 2 h until completely dried at 25 and 37°C, respectively. The dried phage particles were reconstituted in 2 ml phage buffer for 30 min before counting the amount of active phages in the produced phage lysate by the overlay technique, and log reductions of phage counts were calculated. Wild-type T4 phage was used for further experiments to examine the effect of (i) leaving dried phage particles with phage buffer for a longer time, (ii) the addition of polysaccharides, and (iii) lyophilization in the presence and absence of polysaccharides on the phage desiccation stability. The T4 air-dried particles were incubated with the phage buffer for 18 h at 4°C, and the log reduction in the phage count was calculated and compared to that determined after leaving phage particles in buffer for only 30 min. Two hundred microliters of T4 phage with and without maltose (0.1, 0.5, 1, 2, and 5%), trehalose (100 mM), or starch (0.3 and 1.5%) was air-dried until completely dry and then reconstituted in 2 ml of the phage buffer and left for 18 h at 4°C. The log reduction in phage count was calculated and compared to that obtained after air drying the T4 phage without the addition of any polysaccharides. Lyophilization also was studied for drying T4 phages. Two hundred microliters of T4 phage lysate with and without 0.5% maltose, 100 mM trehalose, or 0.3% soluble starch was incubated at −80°C for 30 min and then freeze-dried with a Lyph-lock 6-liter freeze dryer (Labconco, Kansas City, MO) for 22 h at a vacuum pressure of 1.5×10^{-1} Pa. The freeze-dried phage particles were reconstituted in 2 ml of the phage buffer and left overnight at 4°C, and the log reduction in the phage titer was calculated. Each treatment was done in triplicate.

Statistical analysis. The statistical analysis of the experimental data was accomplished with SigmaPlot, version 10.1 (Systat Software Inc., Chicago, IL). A one-way analysis of variance (ANOVA) was performed. In all cases, statistical differences between the means were considered significant when $P < 0.05$.

RESULTS

Charge difference between phage head and tail structures. T4 head and tail components were purified, and the overall charge of each preparation was determined. It was found that the overall charge of the products of the 10^- T4 mutant, which was composed mainly of heads and tail fibers (Fig. 1), was around -5.31 ± 0.67 mV, and that of the 23^- mutant, which was tail and tail fibers, was around 1.80 ± 0.19 mV.

Immobilization of phages on positively charged cellulose membranes. Different concentrations of an *E. coli* phage cocktail in the range of 10^3 to 10^9 PFU/ml that contained EcoM-AG2, EcoM-AG3, and EcoM-AG10 phage were incubated with cellulose membranes that had been modified to carry a positive charge or possessed unmodified surfaces. The esti-

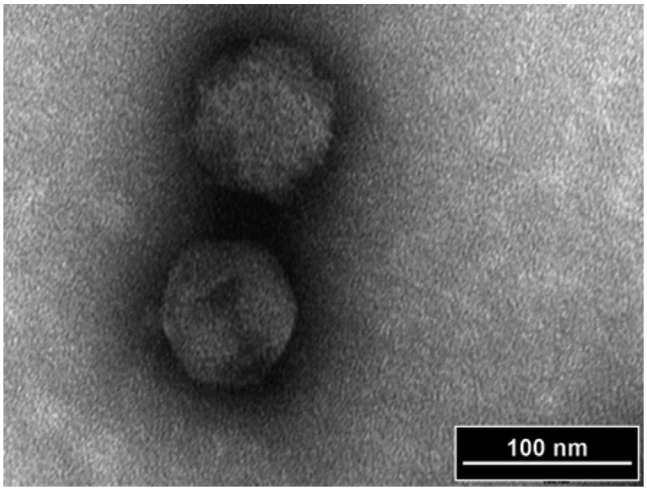

FIG. 1. Transmission electron microscope (TEM) image of T4 heads produced from T4 10^- mutants.

mated percentage of the captured phage particles was 95% for the modified membranes and 71% for the unmodified ones when the initial load was above $\geq 10^5$ PFU/ml; these findings are significantly different ($P < 0.05$). The reduction of the phage count for both modified and unmodified M-3 membranes was ≥ 2 log PFU/ml.

The membranes were washed three times in phage buffer to remove nonspecifically bound phages before being used to cover a lawn of the indicator bacteria. Phage-treated, positively charged cellulose membranes M-9, M-7, M-5, and M-3 developed 646 ± 51, 544 ± 29, 107 ± 10, and 8 ± 1.4 plaques, respectively, which were significantly higher quantities than those developed with the phage-treated, unmodified membranes ($P < 0.05$), where plaque numbers were 106 ± 12 and 68 ± 12 for M-9 and M-7, respectively, with no plaques developing under membranes M-5 and M-3. In another approach to detect the infectivity of the immobilized phages on both positively charged and unmodified membranes, approximately 10^5 CFU/ml *E. coli* O157:H7 (*amp*::*lux*) was added to the membranes, and the effect on bioluminescence signal development was determined. The treatment of cellulose membranes with higher phage cocktail concentrations (M-9, M-7, and M-5) resulted in the nearly complete inhibition of the bioluminescence development at this bacterial concentration (Fig. 2). The M-3 positively charged membrane showed nearly the same bioluminescence pattern as that of the control for 8 h, and then the bioluminescent signal decreased until it reached nearly the same values of samples with higher phage concentrations (M-9, M-7, and M-5) (Fig. 2a). On the other hand, the M-3

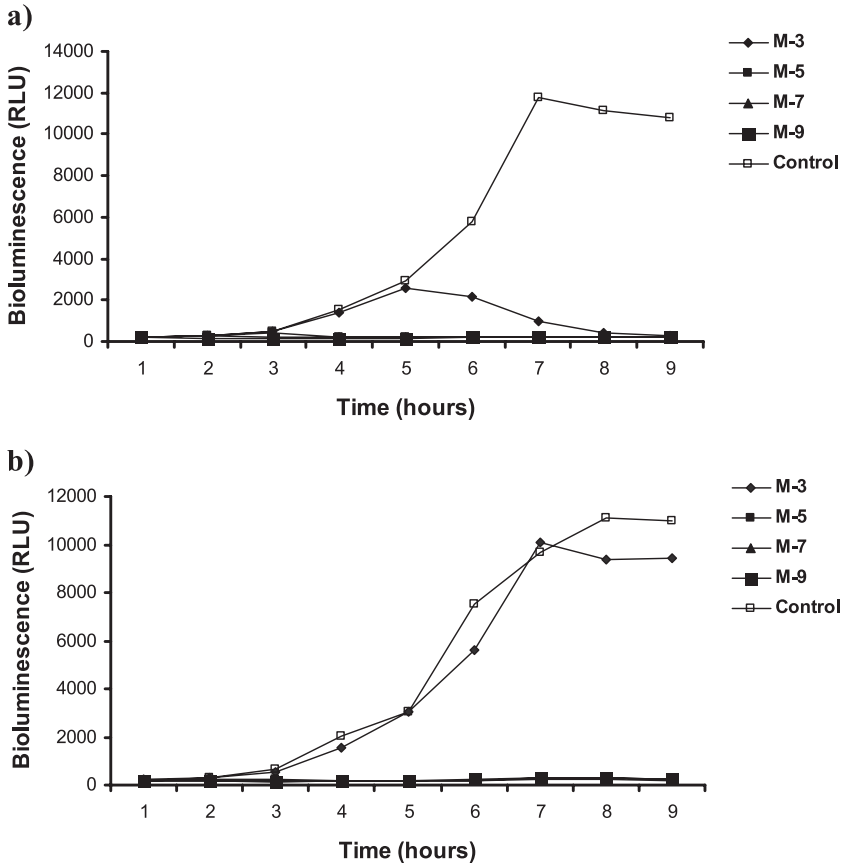

FIG. 2. Bioluminescent signal from *E. coli* O157:H7 (*amp*::*lux*) cells grown with a starting inoculum of around 10^5 CFU/ml in the presence of positively charged (a) and unmodified (b) cellulose membranes treated with different concentrations of *E. coli* phage cocktail. Phage-free membranes were used as the control. Data are the means from three independent replicate trials.

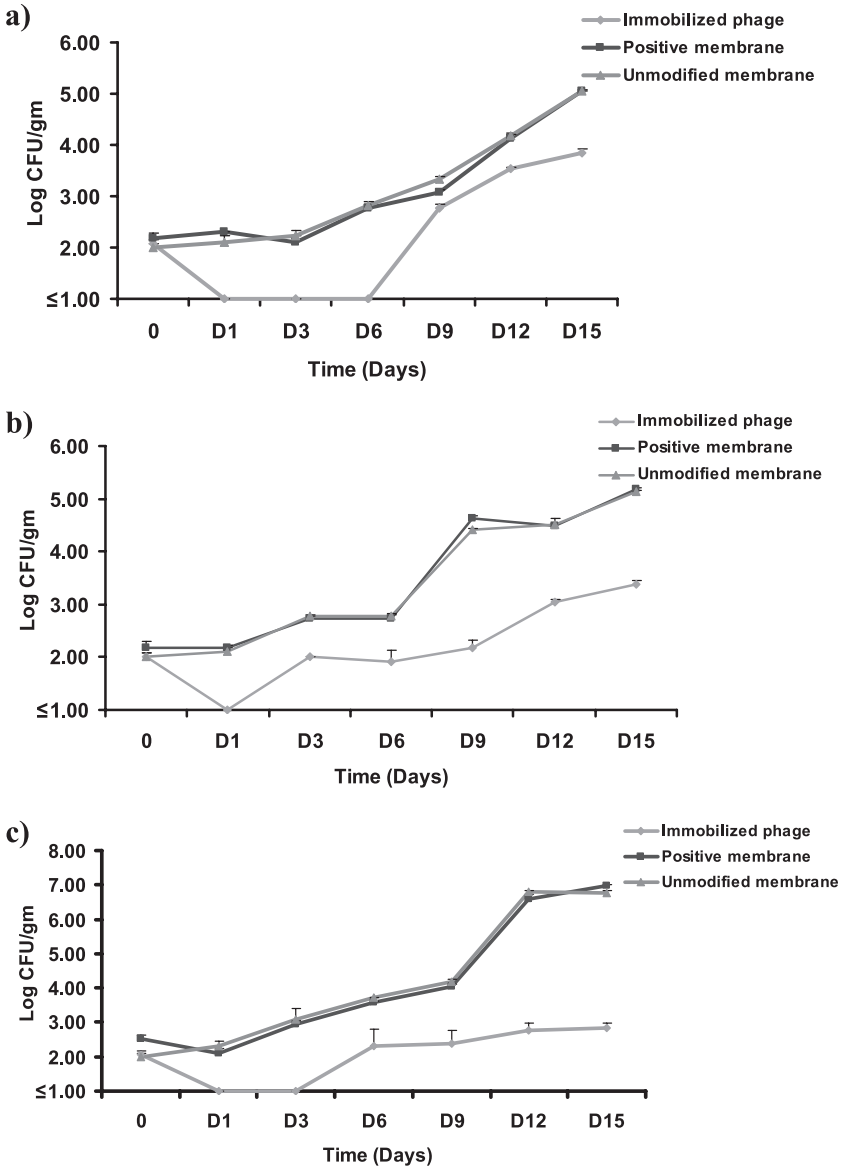

FIG. 3. Effect of the immobilized *Listeria* phage cocktail on growth of *Listeria monocytogenes* C391 on RTE oven-roasted turkey breast incubated at 4°C under aerobic (a), modified atmospheric packaging (MAP) (b), and vacuum (c) conditions. Phage-free positively charged and unmodified cellulose membranes were used as controls. Data are the means from three independent replicate trials.

unmodified membrane resulted in a bioluminescence pattern very similar to that of the control (Fig. 2b).

Potential application of the immobilized phage cocktails on positively charged cellulose membranes to control food-borne pathogens on meat surfaces. *Listeria* and *E. coli* phage cocktails immobilized on positively charged cellulose membranes were examined to control the growth of *L. monocytogenes* and *E. coli* O157:H7 (*amp*::*lux*) in ready-to-eat (RTE) and raw meats, respectively, under different storage temperatures (25, 10, or 4°C) and packaging conditions (aerobic, modified atmosphere packaging, or vacuum). The bacterial count was determined at regular time intervals. It was found that phage-free positively charged membranes had no effect on the growth of the bacteria in all tested cases, with results being similar to those obtained for phage-free unmodified membranes, indicating that neither membrane affected the bacterial growth. When *Listeria*-contaminated RTE oven-roasted turkey breast was incubated aerobically at 25°C in the presence of immobilized *Listeria* phage cocktail, the *Listeria* count was reduced by less than 1 log unit after 6, 12, and 48 h and by around 1.4 log units after 24 h compared to the number of cells recovered from the control samples. At 10°C, count reductions of less than 1 log unit were detected for samples incubated aerobically for 4 days. Interestingly, the level of *L. monocytogenes* was below the detection limit (10 CFU/ml) by the direct plating of the treated samples that were incubated for 6 days at 4°C (Fig. 3a). However, 0.5-, 0.6-, and 1.2-log-unit reductions were detected after 9, 12, and 15 days, respectively, under the same conditions. Incubating immobilized phage-treated meats under MAP conditions at 25°C resulted in bacterial reductions of less than 1 log unit after 6 h and more than 1 log unit at

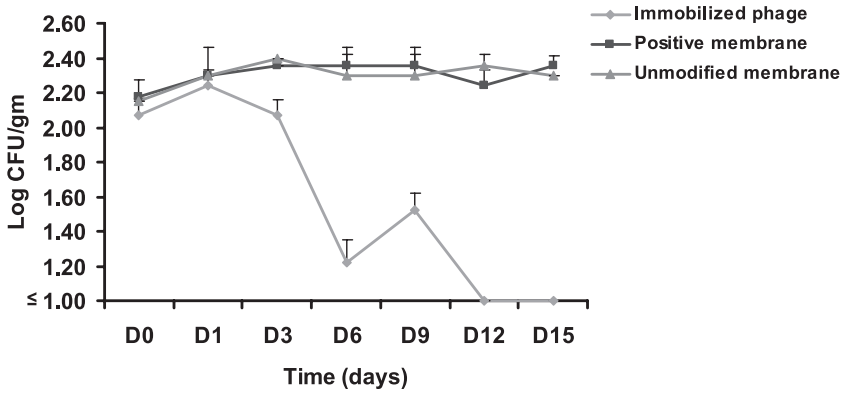

FIG. 4. Effect of the immobilized *E. coli* phage cocktail on the growth of *E. coli* O157:H7 (*amp*::*lux*) C918 on raw beef incubated aerobically at 4°C. Phage-free positively charged and unmodified cellulose membranes were used as controls. Data are the means from three independent replicate trials.

all other sampling times. The highest reduction was after 24 h, when a 1.37-log-unit reduction in count was observed. Incubation at 10°C under the same conditions resulted in a reduction of bacterial cells to an undetectable level after 1 day and an approximately 0.8-log-unit reduction after 4 days. The reduction of *L. monocytogenes* to below the detection limit also was noticed when MAP-packed meats treated with the immobilized *Listeria* phage cocktail were incubated at 4°C for 1 day (Fig. 3b). Approximately 2.2-, 1.48-, and 1.7-log-unit reductions in *Listeria* counts also were observed after 9, 12, and 15 days, respectively. Packing treated RTE meat under vacuum at 25°C in the presence of the immobilized *Listeria* phage cocktail resulted in significant reductions in populations of *Listeria* at all sampling times, with an approximately 1-log-unit reduction after 6 and 12 h and 1.79- and 1.63-log-unit reduction after 24 and 48 h, respectively. At 10°C, the number of recovered cells was below the detection limit after 1 day, and the count was reduced by 2.71 log units after 4 days. With incubation at 4°C, undetectable levels of *L. monocytogenes* were found in the turkey breast after 3 days, and further incubation to 12 and 15 days produced a 4-log-unit reduction in pathogen levels (Fig. 3c).

The immobilized *E. coli* phage cocktail resulted in a nonsignificant reduction in the number of recovered *E. coli* O157:H7 (*amp*::*lux*) cells at all sampling times from artificially inoculated raw beef incubated aerobically at 25°C ($P > 0.05$). However, storing meat samples at 10°C resulted in a reduction of less than a log unit after 4 days. The most pronounced effect was obtained at 4°C, when the number of target bacteria was reduced significantly by about 1 log unit after 6 and 9 days and below the detection limit (10 CFU/ml) by direct plating after 12 and 15 days (Fig. 4). To visually investigate the ability of the immobilized *E. coli* phage cocktail to control the growth of *E. coli* O157:H7 (*amp*::*lux*), images were taken of the bioluminescence that developed on the meat surface after incubation at 10 and 4°C. As can be seen in Fig. 5, the presence of immobilized phage cocktail resulted in the reduction of bioluminescence on the treated meat surfaces.

Effect of drying on the stability of phages. One member of the *Siphoviridae* and four *Myoviridae* phages were used to investigate the effect of drying on the stability of these phages. Phages were air dried and then reconstituted in phage buffer before counting the amount of active phages in the produced phage lysate. There was a significant difference between the log unit reductions of *Siphoviridae* phage SenS-AG11 and the four myoviruses that were air dried at 25 and 37°C ($P < 0.05$) (Fig. 6). Increasing the time of the reconstitution of the dried T4 phage with phage buffer for up to 18 h resulted in a reduction of about 2.96 ± 0.27 log units, which was not significantly different from the 30-min reconstitution time that resulted in a reduction of around 3.05 ± 0.16 log units. The addition of 0.5% maltose and 0.3% starch caused a reduction in the number of inactivated T4 phages by about 0.72 ± 0.22 and 0.76 ± 0.9 log units, respectively, compared to that of the untreated phage. Interestingly, increasing the maltose concentration to 5% caused a greater reduction (about 1.9 ± 0.12 log units) in T4 phage titer after drying. When lyophilization was used to dry T4 phage, the phage count was not significantly reduced after reconstitution in phage buffer ($P < 0.05$). Adding polysaccharides did not result in an improvement in survival compared to that of untreated lyophilized T4 phage ($P < 0.05$).

DISCUSSION

Regulatory acceptance of the use of phage to control foodborne pathogens has triggered the search for new applications for these natural bacterial killers using different strategies to improve consumer and industry acceptance of the technology. One strategy is to immobilize phages on a carrier material, which has been used successfully in detection and phage therapy applications (10, 19, 42). Developing a technique for achieving the oriented immobilization of phages through their heads would enhance their capture efficiency by reducing the amount of nonspecific binding to the matrix.

In this study, the charge difference within the external phage structure provides a strategy to immobilize phage on surfaces through electrostatic interactions. We reported previously that an increase in the overall surface positive charge of silica beads led to an increase in the infective phage binding until the silica surface was saturated with positive charges, at which point the amount of active phage on the material became nearly constant (4). This supported the idea that the tested phages have

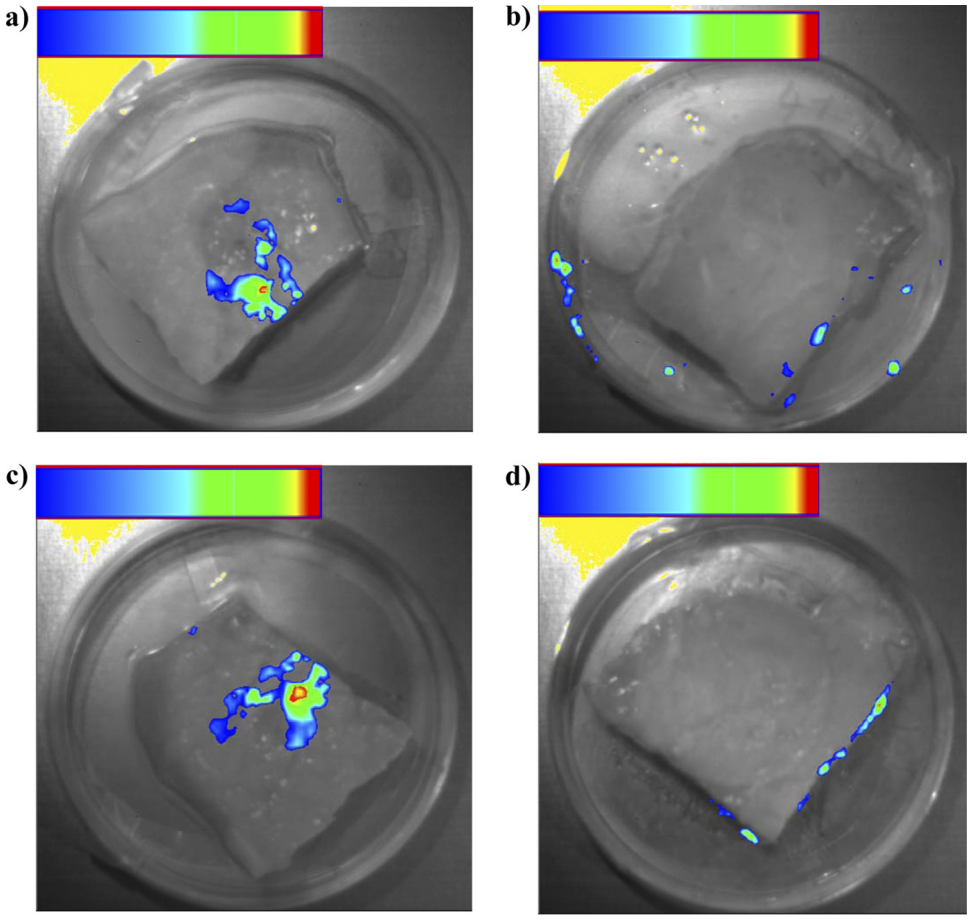

FIG. 5. Bioluminescence activity of *E. coli* O157:H7 (*amp*::*lux*) on the surface of raw beef incubated for 1 week at 10°C (a and b) and 4°C (c and d) and then at 30°C for 16 h. The inoculated meat samples were covered by immobilized *E. coli* phage cocktail on positively charged cellulose membranes (b and d) and phage-free unmodified cellulose membranes (a and c). The blue color represents a low degree of bioluminescence, while red represents the highest level of bioluminescence.

anionic head groups that were interacting with the cationic silica particles.

To further explore this hypothesis, the charge difference between phage heads and tail structures was investigated by Zeta potential measurement. Zeta potential results revealed that the charge of the preparation of T4 heads with tail fibers was net negative, while a preparation containing only tails and tail fibers was positively charged. Serwer and Hayes also suggested that the T7 phage head carries an overall negative charge and that tail fibers are positively charged (35). The pK_a values for major T4 head proteins ranged from 4.62 to 6.63, while that of the tail sheath was 4.80, with a range between 5.21 and 9.76 being reported for tail fiber proteins (5, 17, 36). These earlier data and the results of this study support our conclusion that phage heads exert a net negative charge, which can interact electrostatically with positively charged surfaces. Furthermore, bacterial cell surfaces possess a net negative electrostatic charge on the outer cell envelope due to the ionization of phosphoryl and carboxylate groups which are exposed to the extracellular environment (39). This suggests that the presence of positively charged phage tail fiber proteins helps in the initial attraction of the phage to its host cell wall before receptor interaction.

Building on this knowledge of charge distribution over the phage structure, an *E. coli* phage cocktail was immobilized on modified and unmodified cellulose membranes using the charge difference approach. The initial number of phages attracted to the positively charged membranes was higher than that observed with unmodified surfaces, and this was most apparent with M-9 and M-5 phage-treated membranes. Furthermore, these modified membranes developed a higher number of plaques over a lawn of the indicator bacteria than was seen on unmodified surfaces. The development of plaques underneath the unmodified membranes indicated that a portion of the phages were immobilized in the right orientation, and this was simply due to charge-independent physical adsorption. However, these results indicated that the charge modification obviously enhanced the binding, giving rise to greater numbers of infective phages (i.e., in the right orientation) on the membrane surface among the total number of phages immobilized. This is consistent with recent reports that showed that T4 phage heads were aggregated at pH 5.6 and 7.5 on aminosilanized substrates, where capsids behave as negatively charged entities and are electrostatically attracted to a positively charged surface (1). The charge effect was obvious when the positively charged membranes treated with a low *E. coli* phage cocktail concentration (around 10^3 PFU/ml) were able to reduce the bioluminescence signal from *E. coli* O157:H7 (*amp*::*lux*) compared

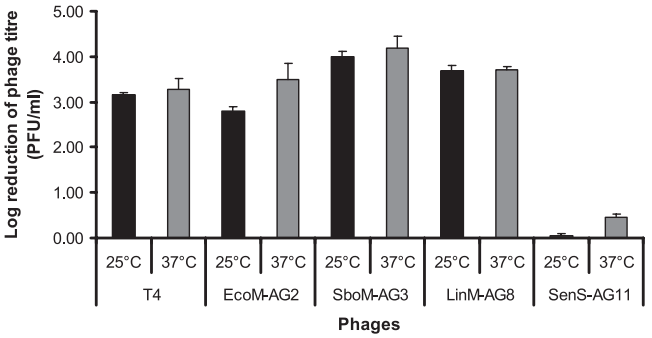

FIG. 6. Effect of air drying at 25 and 37°C on the stability of phages of different morphotypes. Phages were exposed to air at 25 or 37°C until they were completely dry. The dried phage particles were reconstituted in phage buffer before counting the amount of active phages in the produced phage lysate. Data are the means from three independent replicate trials.

to the effect of using an unmodified membrane. The nearly similar inhibitory effect achieved when positively charged and unmodified membranes were exposed initially to a high phage titer suggests that the amount of the immobilized phages in the right orientation on unmodified membranes was enough to provide sufficient multiplicities of infection to achieve an equivalent level of cell lysis. This emphasized that charge-directed immobilization might be helpful to improve the infectivity of less virulent phages. Physical adsorption has been used successfully to immobilize IG40 and Lm P4:A8 filamentous phages on gold surfaces of a quartz crystal microbalance (QCM) biosensor and a surface plasmon resonance (SPR) sensor surface to detect *E. coli* and *L. monocytogenes*, respectively (25, 26). Another filamentous phage specific to *Salmonella* Typhimurium has been physically adsorbed to the surface of a magnetoelastic sensor and used as a biorecognition element for the detection of its host cells at different concentrations (20).

These modified cellulose membranes that contain immobilized phages were able to control the growth of *L. monocytogenes* and *E. coli* O157:H7 in meats incubated at different temperatures and under different packaging conditions. The presence of the immobilized *Listeria* phage cocktail in MAP and vacuum-packed RTE meat resulted in obviously higher reductions in the growth of the target strain in these samples than the reduction observed for aerobically stored samples. A new Health Canada *Listeria* policy for ready-to-eat foods (http://www.hc-sc.gc.ca/fn-an/legislation/pol/policy_listeria_monocytogenes_2011-eng.php) recommends the application of postprocess lethality treatment and/or an antimicrobial agent or process resulting in a final concentration of *L. monocytogenes* of <100 CFU/g at the end of shelf life or measures to control the growth of the organism so that no more than a 0.5-log increase during the shelf life of the RTE food product during challenge studies is achieved. The results of the current study indicate that immobilized *Listeria* phages can be employed in RTE meats packaged under vacuum or MAP to limit the growth of *L. monocytogenes* to less than 0.5 log cycles in RTE foods throughout their shelf lives. This confirms that immobilized phage technology can be used to comply with the revised Health Canada policy.

An immobilized *E. coli* phage cocktail produced a reduction of *E. coli* O157:H7 to undetectable levels when meat was stored at 4°C for 12 and 15 days. The bioluminescence activity of this strain on the meat surface also was remarkably reduced in the presence of the immobilized phage cocktail. This could be explained by either the killing activity of this phage cocktail or the development of phage-resistant bacteria, which affected the expression of genes, including those associated with bioluminescence. O'Flynn indicated that phage-resistant *E. coli* O157:H7 strains are less virulent and had different morphology than the original sensitive strain (27).

Although immobilized bacteriocin and antimycotic agents on cellulose membranes have been used to inhibit the growth of *Listeria monocytogenes* on ham, turkey breast meat, and beef (23, 41) and to increase the shelf life of produce and cheese items (6), this is the first report describing the use of phages immobilized on cellulose membranes to control food-borne pathogens in foods. Although there is no available information on the principle of manufacture, the Eliava Institute in Georgia is using a similar application strategy in phage therapy of infected wounds, which they term PhagoBioDerm (Phage International), in which phages are impregnated on a biodegradable, polymer-based membrane along with ciprofloxacin, benzocaine, and alpha-chymotrypsin (15). This product was able to heal wounds infected with multidrug-resistant *Staphylococcus aureus* within 7 days. Phages also have been immobilized on nylon strips, and this product was found to be effective against most of the major epidemic methicillin-resistant *Staphylococcus aureus* strains (http://www.news-medical.net/?id=8938).

To assist the commercial manufacture of this bioactive membrane, this study investigated the effect of dryness and the use of different ways of drying on phage stability. Interestingly, the tested *Siphoviridae* phage was significantly more tolerant to the effect of air drying than the tested members of the *Myoviridae*. This might be explained by the effect on the contractile sheath, which plays a critical role in the infectivity of *Myoviridae* phages (11). Increasing the time of exposure of the air-dried phage with phage buffer before counting did not help to reactivate T4 phage. In other words, this study suggests that desiccated phages irreversibly lose infectivity. Adding polysaccharides such as 0.5% maltose or 0.3% starch enhanced significantly the tolerance of T4 phage to the air-drying effect. This is consistent with previous reports that mentioned the usefulness of different polysaccharides in enhancing the stability of various biologically active compounds against desiccation (7, 14, 21). This study found that freeze-drying was an efficient method to dry phages with little decrease in phage activity, which would help in developing different paper-coating approaches for the commercial manufacture of phage-containing bioactive membranes. The same technique has been used for the encapsulation of *Staphylococcus aureus* and *Pseudomonas aeruginosa* phages without affecting their lytic activity (29, 30).

Although further studies are needed to develop a final commercial product, these results suggest that it is possible to use the charge difference between phage heads and tail fibers to specifically immobilize phages through their heads, leaving tail fibers free to capture target bacteria and result in infection. The developed bioactive membranes were able to successfully reduce populations of *L. monocytogenes* and *E. coli* O157:H7 in a real food system. This proposed approach can help to broaden phage applications not only to enhance food safety but also in many other fields, such as phage therapy in humans and animals.

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